

Xeroderma Pigmentosum Cells Contain Low Levels of Photoreactivating Enzyme

(DNA repair/ultraviolet light damage/pyrimidine dimers)

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Communicated by R. B. Setlow, October 15, 1974

ABSTRACT Fibroblasts from patients with xeroderma pigmentosum contain low levels of photoreactivating enzyme in comparison to normal cells. Levels vary from 0 (line 1199) to 50 (line 1259) percent of normal. The depressed enzyme levels are not an artifact of low growth rate, age of cell donor, cell culture conditions, assay conditions, the presence of inhibitors, or mycoplasma contamination. We show that human fibroblasts can monomerize pyrimidine dimers *in vivo*.

Xeroderma pigmentosum is a rare disease in which affected individuals develop pigmentation abnormalities and malignant growth in regions of skin exposed to sunlight (1). Cells from most xeroderma patients are deficient in the unscheduled DNA synthesis elicited by ultraviolet light (220-300 nm) in normal cells (2); these cells are thought to lack at least one of the enzymes involved in excision repair of ultraviolet light (UV)-induced damage to DNA (3). However, the existence of xeroderma variants, individuals with apparently normal unscheduled DNA synthesis but with all the clinical manifestations of xeroderma pigmentosum (4), and the limited genetic analysis currently available due to the rareness of the disease, suggested that other factors might contribute to the molecular origin of this sun-induced skin cancer (1).

In photoreactivation, UV-induced cyclobutyl pyrimidine dimers in DNA are repaired by the photoreactivating enzyme in a two-step reaction: the enzyme binds to a dimer-containing region of DNA, and in the presence of light of wavelengths between 300 and 600 nm monomerizes the dimer, thus restoring biological activity to the DNA (5, 6). Until recently, the photoreactivating enzyme was thought to be absent from placental mammals (7, 8). However, since Sutherland (9) has recently demonstrated the presence of the enzyme in human leukocytes, and Sutherland *et al.* (10) have found the enzyme in murine and human fibroblasts, we have examined the level of photoreactivating enzyme in cells of xeroderma patients.

Xeroderma cells contain less photoreactivating enzyme than do normal fibroblasts; the activity ranges from 0 to 50% of the normal lines. We show that the lower levels of photoreactivating enzyme are not artifacts of assay conditions, cell culture, or growth conditions, or contamination. We also present a direct demonstration of photoreactivation of pyrimidine dimers in human cells *in vivo*. We predict that individuals with normal levels of excision repair but low total DNA repair capacity due to a low photoreactivating enzyme level may well have a high probability of developing sunlight-induced skin malignancies.

MATERIALS AND METHODS

Cells and Cell Growth. Xeroderma pigmentosum lines CRL 1223 (Jay Tim), CRL 1199 (Po Co), CRL 1200 (Te Ko), and CRL 1259 (XP2) were obtained from the American Type Cul-

ture Collection; lines CRL 1161 (Ge Ar) and CRL 1204 (Gor Do) were the gift of Dr. R. Teplitz, City of Hope Medical Center, who had obtained them from ATCC. Normal lines were obtained as follows: NHF, human neonatal foreskin fibroblasts, from Dr. J. Clark, College of Medicine, UCI; HESM (human embryonic skin and muscle fibroblasts) from Flow Laboratories; CRL 1222 (El San), CRL 1126 (Dor Son), CRL 1147 (Ter Loy) and CRL 1229 (Le San) from ATCC. Cells were grown in Dulbecco's Modified Minimal Eagle's Medium plus 20% fetal calf serum in Falcon plastic T-75 flasks. General methods for cell culturing and periodic assay for bacterial contamination have been described (11).

Preparation of Extracts and Photoreactivation Assays. Each flask was washed three times with 30 ml of 0.15 M NaCl, and the cells were scraped into 2 ml of 0.15 M NaCl and centrifuged at $500 \times g$ for 5 min. The cells were washed three times by suspension in 2 ml of 0.15 M NaCl and centrifugation as before. The washed cell pellet was drained and then resuspended in Buffer E [10 mM Tris·HCl (pH 7), 0.1 mM dithiothreitol, 0.1 mM EDTA]. The cells were sonicated for 45 sec in a Kontes sonicator.

The assay for photoreactivation has been described in detail (12). Briefly, photoreactivating enzyme is added to an assay mixture (0.2 ml) containing 20 mM phosphate buffer (pH 7.2), 0.1 mM dithiothreitol, 0.1 mM EDTA, plus 30-100 pmol of ^{32}P -labeled, T7 bacteriophage DNA that had been exposed to 300 J/m² of ultraviolet radiation from a low-pressure Hg bulb.

Assay mixtures were prepared in duplicate; after 5 min of preincubation at 37° in a circulating water bath, one tube was exposed to photoreactivating light from a 150 W spot lamp, while the other was placed in the dark. The samples were digested to a mixture of ^{32}P -labeled, dimer-containing oligonucleotides, nucleosides, and inorganic $^{32}\text{P}_i$ (13) by the sequential addition of 25 μg of DNase I, followed by a mixture of 100 μg of venom phosphodiesterase and 1 μg of alkaline phosphatase, plus 10 μl of 1 M Tris, pH 8.

Oligonucleotides were separated from inorganic phosphate by adsorption to Norit; the ^{32}P on Norit is a linear function of the dimer content of the DNA. Photoreactivating enzyme activity is calculated by subtracting the counts on Norit of the sample exposed to photoreactivating light from the counts on Norit of the duplicate mixture kept in the dark. Units of photoreactivating enzyme activity are pmol/mg per hr. Protein concentrations were determined by the Lowry method (14), with a bovine serum albumin standard.

Tests for Mycoplasma. Cells were tested for mycoplasma contamination by the sucrose gradient sedimentation technique of [^3H]thymidine-labeled cultures (15). Confluent cells

TABLE 1. Levels of photoreactivating enzyme and unscheduled synthesis in normal and xeroderma cells

| Line | Description | Age at biopsy | Xeroderma complementation group* | Unscheduled synthesis (% of normal) | Photoreactivating enzyme activity | |
|--------------------|-----------------------------------|---------------|----------------------------------|-------------------------------------|-----------------------------------|------|
| | | | | | Units | % |
| NHF | Human foreskin fibroblasts | Neonatal | — | (100)† | 627‡ | 100 |
| HESM | Human skin and muscle fibroblasts | Embryonic | — | (100)† | 625 | 99 |
| CRL 1199 (Po Co) | Xeroderma pigmentosum | 28 yr | B | 3–7 | 0 | 0 |
| CRL 1200 (Te Ko) | Xeroderma pigmentosum | 11 yr | D | 25–55 | 50 | 8 |
| CRL 1161 (Ge Ar) | Xeroderma pigmentosum | 12 yr | C | (10–25)† | 99.6 | 15.8 |
| CRL 1223 (Jay Tim) | Xeroderma pigmentosum | 7 yr | A | <2 | 227 | 36.3 |
| CRL 1259 (XP 2) | Xeroderma pigmentosum | 34 yr | E | † | 311.1 | 49.5 |

* See ref. 1.

† Although lines 1161, NHF, and HESM have not been tested for unscheduled synthesis, the values shown are representative of complementation group C and normal lines, respectively. Line 1259, the sole representative of complementation group E, has not been tested for unscheduled synthesis. For further discussion of unscheduled synthesis, see ref. 1.

‡ Typical ranges of enzyme activity were $\pm 5\%$ for samples with high activity and $\pm 10\%$ for those of lower activity. (See ref. 12 for further discussion.)

were incubated for 20 hr with 15 $\mu\text{Ci}/\text{ml}$ of [^3H]thymidine (specific activity 20 Ci/mmol). A second test for mycoplasma was the determination of ratio of [^3H]uracil to [^3H]uridine incorporation (16).

Dimer Photoreactivation In Vivo. Jay Tim (CRL 1223) cells on glass coverslips were labeled overnight with tritiated thymidine at an activity of 5 $\mu\text{Ci}/\text{ml}$. Cells were washed with 0.15 M NaCl and the coverslips placed in a petri dish and covered with 0.15 M NaCl. Cells were exposed to 10 J/m² [as measured with a Jagger (17) meter calibrated with a Hewlett-Packard thermopile] of 254-nm light from a germicidal lamp. One sample was harvested immediately; other samples were placed in separate bottles, covered with medium, placed in a Plexiglas chamber at 37°, and exposed to photoreactivating light from an incandescent bulb (60 W) at about 15-cm distance for 5 min, 30 min, 1 hr, or 24 hr. Other UV-irradiated samples were kept in foil-wrapped bottles for 30 min and 24 hr. Two samples that had not been exposed to ultraviolet light were exposed to photoreactivating light for 30 min or 24 hr.

The cells were harvested by scraping them into 0.15 M NaCl, and adding 2 nmol of carrier calf thymus DNA plus ice-cold trichloroacetic acid to a final concentration of 20%. After 15 min on ice, the samples were centrifuged at $27,000 \times g$ for 15 min, drained briefly, and hydrolyzed in 98% formic acid in sealed vials at 175°C for 30 min. The formic acid was removed by vacuum evaporation, and the samples were chromatographed on Whatman no. 1 paper in butanol-acetic acid-water (40:6:15, v/v/v) by the method of Setlow *et al.* (18). Chromatograms were sliced, and counted in a liquid scintillation counter.

RESULTS

Leukocytes and skin fibroblasts from normal humans contain high levels of photoreactivating enzyme (9, 10). We thus examined fibroblasts from patients with xeroderma pigmentosum, the hereditary disease in which sunlight-exposed areas of skin develop malignancies. Table 1 shows that the xero-

derma cell lines indeed have depressed levels of photoreactivating enzyme, ranging from CRL 1199, with no detectable enzyme activity, to CRL 1259, with about 50% of the normal level. There is no direct relationship between excision repair capacity, as measured by unscheduled synthesis incorporation (see ref. 1) and levels of photoreactivating enzyme; unscheduled synthesis ability increases in the order 1223 < 1199 < 1161 < 1200, while photoreactivating enzyme levels show an increasing series 1199 < 1200 < 1161 < 1223 < 1259. Nor is there an obvious relationship between age at biopsy and photoreactivating enzyme level: although Jay Tim (CRL 1223), who was biopsied at 7 years, had 36% of the normal enzyme level, the highest enzyme level was found in CRL 1259, biopsied at 34 years, and, thus, the xeroderma line derived from the oldest individual tested.

We have also determined that the low levels of photoreactivating enzyme in xeroderma pigmentosum patients cannot be accounted for by the following: (i) *Low growth rate and general depression of all enzyme levels.* It is possible that low growth rates of xeroderma cells compared to normal lines might lead to a general depression of all enzymes, including the photoreactivating enzyme. Two lines of evidence indicate that this is not the case. First, although the growth curves shown in Fig. 1 indicate doubling times of 39 hr and 23.5 hr for the NHF and HESM normal lines, respectively, both lines contain approximately the same amount of photoreactivating enzyme. The xeroderma line CRL 1200 has a doubling time of 34.8 hr, faster than the NHF, even though it contains only 8% as much enzyme activity. Thus, there is no direct correlation between growth rate and level of photoreactivating enzyme, and the low level of photoreactivating enzyme in the xeroderma cells is not due to slower growth of these lines. Second, levels of pyruvate kinase in the normal NHF line and the slowly growing xerodermoid 1161 line (see Fig. 1) were determined: the normal cells contained 5.66×10^{-8} U/mg, while the xeroderma cells contained 5.22×10^{-8} U/mg, a comparable enzyme level. Since pyruvate kinase, an important enzyme of carbohydrate metabolism, is present at

TABLE 2. Photoreactivating enzyme in normal cells

| Cell line | Age at biopsy (years) | Photoreactivating enzyme activity | |
|--------------|-----------------------|-----------------------------------|-----|
| | | Units | % |
| NHF | Neonatal | 625 | 100 |
| 1222 El San | 8 | 701 | 112 |
| 1229 Le San | 33 | 670 | 107 |
| 1126 Dor Son | 73 | 469 | 75 |

roughly the same levels in these cells, we conclude that the xeroderma lines do not show general depression of all metabolic pathways.

(ii) *Photoreactivating enzyme and age of cell donor.* Photoreactivating enzyme levels are higher in juveniles than in the adults of some species (see ref. 8). If this is true in humans, the low levels of photoreactivating enzyme in the xeroderma lines might merely be due to the increased age at biopsy relative to the neonatal and embryonic normal lines. However, as Table 1 shows, within the xeroderma lines, there is no direct correlation of age and enzyme level: CRL 1200 (11 years) contains only 8% of the normal enzyme level, and CRL 1259 (34 years) contains five times more photoreactivating enzyme. We have also examined the enzyme content in cells taken from normal humans at 8, 33, and 73 years. Table 2 shows that there is no direct correlation of age and enzyme level, and that even cells from the 73-year-old donor contained reasonably high levels of photoreactivating enzyme.

(iii) *Photoreactivating enzyme in cultured cells.* Many enzymes expressed in the intact animal are not present in cultured cell lines derived from that animal. If the xeroderma lines were of higher passage number than the normal lines, this might account for the lower enzyme levels. We thus examined first the question of loss of photoreactivating enzyme in human fibroblasts. Table 3 shows the results of this determination for the two normal lines NHF (received as a high passage line) and HESM (received as low passage cells). In the high passage line, photoreactivating enzyme activity remained at roughly the same level over a span of 22 passages. In the low passage line, no change in photoreactivating enzyme was detected for the nine passages the cells were tested. These data indicate that no loss of photoreactivating enzyme was observed in either the low or high passage lines. The xeroderma lines, received from ATCC as third or fourth passage lines, showed no decrease in photoreactivating enzyme activity during the course of these experiments. Since the xeroderma lines were assayed at roughly the same passage

TABLE 3. Photoreactivating enzyme activity and cell passage

| Cell line | Cell passage | Photoreactivating enzyme activity (%) |
|--------------------|--------------|---------------------------------------|
| NHF (high passage) | +3 | 100 |
| | +4 | 94.6 |
| | +6 | 101 |
| | +8 | 120 |
| | +22 | 102 |
| HESM (low passage) | +1 | 100 |
| | +9 | 112 |

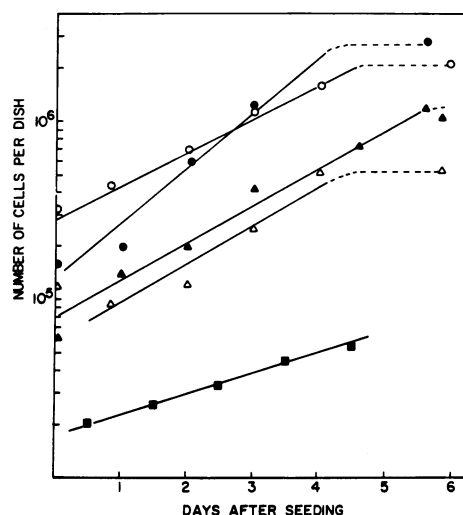


FIG. 1. Growth rate of five representative fibroblast lines. Cells were seeded into plastic flasks, and their number was determined at 24-hr intervals by trypsinization, staining with a vital dye, and counting. Fresh medium was supplied every 48 hr. Lines examined were the two normal lines, NHF (○) and HESM (●), and three xeroderma lines, 1200 (▲), 1223 (△), and 1161 (■).

number as were the normals, and since no loss of enzyme activity could be detected in the normal lines, we conclude that the low enzyme activity in the xeroderma cells is not an artifact of cell culture.

(iv) *Optima of the human fibroblast enzyme.* Xeroderma cells might contain functional photoreactivating enzyme that had different optima of pH or ionic strength or different metal requirement from the normal fibroblasts or leukocytes. We thus tested xeroderma line 1161 at lower (6.8) and higher (7.5) pH values than the optimum (7.2), but found a decrease in measured activity (Table 4, lines 2–4). At lower (0.03) ionic strength, the enzyme activity in the 1161 cells was about the same as under standard conditions ($\mu = 0.06$), but at higher ionic strength (0.123), the activity was decreased (Table 4, lines 5–6). Neither the presence nor absence of magnesium affected the enzyme activity. The photoreactivating enzyme

TABLE 4. Optimal conditions for the human fibroblast enzyme

| | pH | MgCl ₂ (M) | NaCl (M) | Total ionic strength | Cell line | Photoreactivating enzyme activity (%) |
|----|-----|-----------------------|----------|----------------------|-----------|---------------------------------------|
| 1* | 7.2 | 0.01 | 0 | 0.06 | NHF | 100 |
| 2 | 6.8 | 0.01 | 0 | 0.068 | 1161 | 0–5† |
| 3 | 7.2 | 0.01 | 0 | 0.06 | 1161 | 15 |
| 4 | 7.5 | 0.01 | 0 | 0.059 | 1161 | 0–5† |
| 5 | 7.2 | 0 | 0 | 0.03 | 1161 | 17 |
| 6 | 7.2 | 0.01 | 0.0625 | 0.123 | 1161 | 0–5† |

* Optimal conditions for photoreactivating enzyme in normal human fibroblasts are 0.02 M phosphate buffer (pH 7.2), 0.01 M MgCl₂, 0.1 mM dithiothreitol, 0.1 mM EDTA. All other assay mixtures contain these components unless otherwise stated.

† Activity was so low in these cases that very low levels could not be reliably distinguished from complete absence of activity.

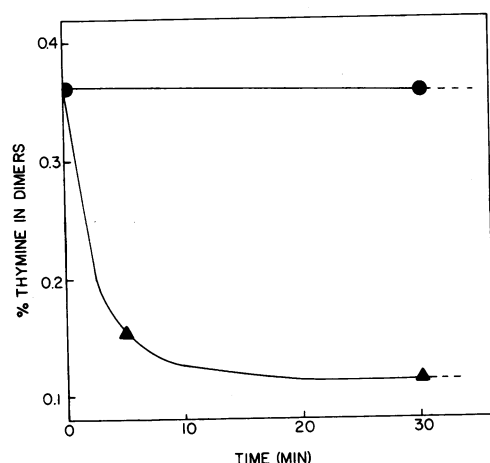


FIG. 2. Photoreactivation of pyrimidine dimers in human cells. Cells of CRL 1223 were grown in monolayers on coverslips labeled with [^3H]thymidine, rinsed, and exposed to 10 J/m^2 of 254-nm radiation. One sample was harvested immediately after UV irradiation (●), two were exposed to photoreactivating light (▲), and one was kept in the dark (●). The samples were harvested, and the dimer content in the DNA was determined. In a parallel experiment, samples were exposed to photoreactivating light for 1 hr or 24 hr; no further reduction in dimer content could be detected. In the dark, the dimer content of the DNA was only slightly decreased after 24 hr.

from xeroderma cells has optima of pH and ionic strength similar to the enzyme from normal cells, and, thus, the low activity is not an artifact of assay conditions.

(v) *Inhibitors from xeroderma cell extracts.* Xeroderma cells might contain normal levels of photoreactivating enzyme that would not be detected because of an inhibitor of the enzyme present in xeroderma extracts but not in those of normal cells. We thus designed mixing experiments to test for the presence of such inhibitors: extracts of xeroderma cell lines 1161 or 1199 had neither stimulatory nor inhibitory effects on the activity of normal fibroblast enzyme. We can thus find no evidence for the existence of inhibitors of the photoreactivating enzyme in xeroderma cell extracts.

(vi) *Mycoplasma contamination.* Since mycoplasma can be photoreactivated (19), the enzyme involved might be one coded by the mycoplasma genome and might artificially inflate the apparent photoreactivating enzyme activity in the normal lines. We thus tested the normal lines for the presence of mycoplasma in two ways. We labeled the cultures overnight with a high concentration of [^3H]thymidine and attempted to detect radioactive mycoplasma in the region of sucrose density corresponding to their density (15). No evidence was found for any DNA-containing material in this density range. This result was confirmed by the [^3H]uracil to [^3H]uridine incorporation method (16).

Photoreactivation of Pyrimidine Dimers In Vivo. Photoreactivating enzyme levels can affect UV-induced cell killing, mutation, or transformation only if the enzyme can act on dimers in the DNA of intact human cells. Since photoreactivation of dimers in living mammalian cells has not been demonstrated (see *Discussion*), we examined the ability of CRL 1223 to photoreactivate dimers in its DNA. [CRL 1223 was chosen because of its low excision repair capacity (see ref. 20 for a discussion of detection of photoreactivation in excision

proficient and excision deficient cells), its rapid growth rate (Fig. 1), and its relatively high residual photoreactivating enzyme level (Table 1).] Fig. 2 shows that CRL 1223 cells efficiently remove dimers from their DNA when exposed to photoreactivating light; in the dark, the dimers remain in the DNA. These results constitute a direct demonstration of the ability of the human photoreactivating enzyme to catalyze dimer monomerization in living cells. Although all the dimers were not apparently photoreactivated by the enzyme, the sizeable background of radioactive material appearing in the dimer region of the chromatogram in unirradiated cells makes difficult an exact determination of the photoreactivable sector.

DISCUSSION

Cells from xeroderma pigmentosum patients are deficient in UV-induced unscheduled DNA synthesis (2) and are thought to lack at least one of the enzymes involved in excision repair of DNA (3). Xeroderma cells also contain lower levels of photoreactivating enzyme than do cells from normal individuals. We have shown that the low enzyme levels are not the result of slower growth, general depression of all enzyme levels, donor age, culture passage number, unfavorable assay conditions, presence of inhibitors, or mycoplasma contamination.

If these low levels of photoreactivating enzyme are biologically significant, the enzyme must play an active part in DNA repair in intact human cells. What is the evidence for the presence of photoreactivating enzyme and its active role in repair in placental mammals? Although Cook and McGrath (7) were unable to find the enzyme in extracts of placental mammals, Sutherland (9) showed that under the proper conditions of pH and ionic strength, a photoreactivating enzyme could be demonstrated in human leukocytes. Sutherland *et al.* (10) have found that the enzyme purified by Sutherland (9) was a true photoreactivating enzyme. Since the data on the role of the enzyme in repair in living cells are contradictory (23–26) and subject to possible experimental difficulties (see refs. 20 and 21), we examined the photoreactivation of [^3H]thymine-labeled dimers in the DNA of the xerodermoid line Jay Tim. In the presence of photoreactivating light, dimers were rapidly removed from the cellular DNA, although they remained in the DNA when the cells were kept in the dark. This evidence indicates that the photoreactivating enzyme is indeed active in DNA repair in human cells.

If, then, the low levels of photoreactivating enzyme are a true property of xeroderma cells, what might be the biological basis for the decreased enzyme levels? The reduction of photoreactivating enzyme might result from a mutation in a gene controlling both photoreactivating enzyme and the excision enzymes; such control systems have been proposed for eukaryotes by Davidson and Britten (22). This model predicts that the affected repair enzymes should be unaltered in sequence and structure and the only change should be the amount of that enzyme produced in the cell. The low levels of photoreactivating enzyme and the excision enzyme(s) might also result from independent mutations leading to inadequate repair of UV-induced lesions. Thus, xeroderma patients might be deficient in more than one repair pathway. If the normal individual is EP (where E signifies the presence of the entire excision pathway, and P the presence of photoreactivating enzyme), individuals afflicted with xeroderma might be ep (where e and p indicate decreased level of at least one excision enzyme and of the photoreactivating enzyme, respectively).

The significant effect would be the reduction of the DNA repair capacity beyond a critical repair threshold, the value of which would depend on the ultraviolet light burden to the individual. In the case of a patient with no excision repair or photoreactivating enzyme, that value would be low indeed. This model makes two predictions: (i) enzymes present at low levels would probably be altered in sequence or structure (unless the mutation were in a untranscribed promotor site), and (ii) there should exist individuals with propensity for sunlight-induced skin cancer who have a decreased total DNA repair capacity resulting from mutation in any one of the repair pathways. The two models of the molecular origin of xeroderma are nonexclusive; some xeroderma cases may result from control mutations, if indeed the Davidson-Britten model is applicable to DNA repair genes, and others may be the result of mutations in structural genes. Since we show that the photoreactivating enzyme does function in DNA repair in human cells, it is tempting to speculate that its absence in xeroderma patients may contribute to their development of sunlight-induced skin malignancies. However, an accurate assessment must await the determination of the range of DNA repair capacities in normal individuals and in patients with sunlight-induced skin malignancies.

We thank Dr. Daniel Wulff, who suggested that xeroderma cells might lack photoreactivating enzyme, and Dr. Barbara Hamkalo for helpful discussions; and Drs. J. Clark and R. Teplitz for their gift of several cell lines. We especially thank Dr. K. Ibsen for performing the pyruvate kinase assays. This research was supported by Grants USPHS CA-14005 to B.M.S. and USPHS CA-11861 to E.K.W.

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